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14. ABSTRACT
Patients with Neurofibromatosis (NF1) exhibit deficient bone healing/ The cause of poor bone healing in NF1 is unclear, and pharmacologic approaches to improve bone repair are lacking. Beta-catenin is a protein that regulates osteoblasts during bone healing. When beta-catenin protein level is high, it prevents osteoblast differentiation, and undifferentiated fibroblast-like cells persist at the fracture site, resulting in a pseudarthrosis. Genetically engineered mice in which the Nf1 gene can be deleted when cells are exposed to Cre-Recombinase were studied. An adenovirus expressing Cre-Recombinase was injected to the fracture site to knock out the gene. Mice in which the beta-catenin gene can be knocked out by exposure to Cre-Recombinase were used to decrease beta-catenin during fracture repair. An open tibial fracture, fixed with an intramedullar pin, was used to study fracture healing. Five to ten mice were studied in each group at each time point, and fracture repair assessed at three and six weeks using radiology and histology. Beta-catenin protein level during fracture repair in mice lacking the Nf1 gene measured five times higher than normal. Mice lacking Nf1 gene showed deficient fracture repair. In contrast, mice lacking Nf1 gene, but that also express a low level of beta-catenin healed their tibia fracture quicker and with more bone as measured using both radiographic and histologic parameters. This work so far shows that beta-catenin protein is elevated during fracture repair in mice lacking Nf1. Inhibition of beta-catenin can improve the quality of the bone repair process.

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INTRODUCTION:

This is the final report of the work performed over two-years.

Patients with Neurofibromatosis (NF1) exhibit deficient bone healing. The cause of poor bone healing in NF1 is unclear, and pharmacologic approaches to improve bone repair are lacking. Beta-catenin is a protein that regulates osteoblasts during bone healing. When beta-catenin protein level is high, it prevents osteoblast differentiation, and undifferentiated fibroblast-like cells persist at the fracture site, resulting in a pseudarthrosis. Genetically engineered mice in which the Nf1 gene can be deleted when cells are exposed to Cre-Recombinase were studied. An adenovirus expressing Cre-Recombinase was injected to the fracture site to knock out the gene. Mice in which the beta-catenin gene can be knocked out by exposure to Cre-Recombinase were used to decrease beta-catenin during fracture repair. An open tibial fracture, fixed with an intramedullar pin, was used to study fracture healing. Five to ten mice were studied in each group at each time point, and fracture repair assessed at three and six weeks using radiology and histology. Beta-catenin protein level during fracture repair in mice lacking the Nf1 gene measured five times higher than normal. Mice lacking Nf1 gene showed deficient fracture repair. In contrast, mice lacking Nf1 gene, but that also express a low level of beta-catenin healed their tibia fracture quicker and with more bone as measured using both radiographic and histologic parameters. Furthermore, treating mice with the drug nefopam, which was previously shown to target beta-catenin, improved the quality of bone healing in Nf1 deficient mice, This work shows that beta-catenin protein is elevated during fracture repair in mice lacking Nf1. Inhibition of beta-catenin either genetically or pharmacologically can improve the quality of the bone repair process.

OVERALL PROJECT SUMMARY

Roughly half of patients with Neurofibromatosis type one (NF1) have abnormalities of their bones. In severe cases, the bones “melt away” or will fail to heal following even the most trivial trauma. This occurs frequently in the tibia, where it is seen in early childhood and is termed congenital pseudarthrosis of the tibia (CPT) or tibial dysplasia. CPT is difficult to treat, and in severe cases results in amputation. When this process occurs in the bones of the spine, it leads to rapidly progressive spinal deformity, which can cause paralysis.

Long bones develop from a cartilaginous template or anlage through a process termed endochondral ossification. At the centre of the template, blood vessels invade the cartilage matrix, bringing osteoblasts to produce bone. Cartilage persists at the ends of the bone, as a growth plate, is responsible for longitudinal bone growth. Osteoblasts derive from mesenchymal progenitor cells, termed MSCs (mesenchymal stem or stromal cells). MSCs can be derived from a number of sources and they exist in bone marrow as stromal cells. The relative numbers of mesenchymal progenitors in bone marrow can be identified as Colony Forming Units-Fibroblastic (CFU-F), while progenitor cells that differentiate to osteoblasts are identified as CFU-Osteoblastic (CFU-O). This developmental process is recapitulated during fracture repair, although the liberation of growth factors from damaged matrix and cells, as well as from inflammatory cells recruited to the fracture site initiate the repair process.

The NF1 protein product is a negative regulator of Ras signaling and is expressed at low levels in osteoblasts and chondrocytes, as well as in osteoprogenitor cells. Ras signaling impacts cells regulating bone development and homeostasis, by inhibiting osteoblast numbers and activity, as well as activating bone resorption by activating osteoclasts. The cause of pseudarthrosis in the bones in NF1 is unclear, although in human samples, loss of the wild type *NF1* allele, hyperproliferation of fibrous tissue, and osteoclast activation in the area of a fracture has been reported. Pseudarthrosis of the tibia is modeled in mice by examining tibia fracture repair in mice expressing *Nf1* floxed (fl) alleles. To activate the conditional alleles in fracture repair infection with an adenovirus-expressing cre-recombinase (Ad-cre) is used. With this approach, data from our lab and others shows that recombination is effective driven in most cells at the fracture repair site. Analysis of tibia fracture healing in $Nf1^{fl/fl}$ mice treated with Ad-cre shows a hyperproliferation of undifferentiated mesenchymal cells at the fracture site[1]. There is also a less severe, but similar fracture phenotype in $Nf1^{+/-}$ mice. Small case series suggests the use of bone morphogenic protein as a locally applied biologic, or the use of bisphosphonate medications to inhibit osteoclasts will facilitate the bone repair process in patients with NF1. Except for these case series, there are no treatments that are reported to improve bone health or fracture repair in NF1, and importantly, no medications that stimulate osteogenesis in this condition.

One pathway that is critical to osteoblast differentiation is the canonical Wnt signalling pathway. This is one of several pathways activated by Wnt ligands. Signalling is initiated when LRP and Frizzled receptor are activated by an appropriate secreted Wnt protein. There are also antagonists of canonical signaling, including Dickkopf (DKK) proteins. β-catenin is a critical mediator in the canonical pathway, and in the absence of an appropriate ligand, it is phosphorylated by a multi-protein “destruction” complex, resulting in its ubiquitination and proteosomal degradation. In the presence of an appropriate Wnt ligand, β-catenin is not degraded, translocates to the nucleus, where in concert with members of the T-cell-factor / Lymphoid-enhancer-factor (Tcf/Lef) family, activates transcription. The target genes are cell type specific, but *AXIN2* is a target in most cell types.

Data from our work on bone fracture repair in mice, and from several other groups, indicates that the canonical Wnt/β-catenin pathway promotes osteoblastic cell proliferation and differentiation in mesenchymal progenitors (MSCs). However, the level of β-catenin activity is important. In the early phases of MSC differentiation to osteoblasts, β-catenin needs to be precisely regulated to allow osteogenesis, and elevated levels inhibit osteogenesis. Genetic and pharmacologic studies show that elevated levels prevent normal bone fracture repair. This is an important concept targeting this pathway therapeutically, as in situations in which β-catenin levels are elevated further activation will hinder osteogenesis. Interestingly, data from our lab shows that mice in which we can conditional stabilized β-catenin alleles are activated by Ad-cre in a tibia fracture shows a phenotype reminiscent of that seen in $Nf1^{fl/fl}$ mice[2].

Since β-catenin plays a critical role in osteoblast differentiation and fracture repair, and the fracture repair phenotype of mice in which β-catenin is dysregulated is similar to that in mice deficient in *Nf1*, we examined how β-catenin is regulated during osteoblast differentiation in neurofibromatosis, and how its modulation could be used to improve healing in patients with neurofibromatosis.

To identify agents that inhibit beta-catenin signaling, we undertook a high throughput screen using mesenchymal cells exhibiting hyperactive β-catenin signaling to identify an agent that would target β-catenin signaling. The screen identified Nefopam (5-methyl-1-phenyl-1,3,4,6-tetrahydro-2,5-benzoxazocine). Nefopam is a centrally-acting but non-opioid analgesic drug of the benzoxazocine chemical class which was developed in the 1970s by cyclization of diphenhydramine. It has an analgesic effect that is mildly stronger than aspirin, but not as strong as codeine, and has few side effects. The mechanism of action of Nefopam is not completely elucidated, although inhibition of serotonin, regulation of dopamine and noradrenaline reuptake, and the regulation histamine H3 receptors and glutamate are all hypothesized to play a role in its analgesic effect. We tested Nefopam in-vivo in reparative processes and found that treatment resulted in a 50% decline in beta-catenin levels in-vivo[3]. To test a pharmacologic approach to the modulation of beta-catenin, mice will be treated with Nefopam. Tibial fractures were established in *Nf1*$^{fl/fl}$ mice, the conditional alleles were activated by infection with Ad-cre. Mice were treated with Nefopam (40mg/kg body weight – a dose we previously found inhibits beta-catenin in mice[3]), or carrier (0.1% DMSO) administered daily by oral gavage. Treatment was started at the time the fracture is generated, and continued until the time of sacrifice.

Body

Task 1. Compare the levels of β-catenin and its transcriptional activation during fracture repair in mice deficient in *Nf1*.

1a. Breed *Nf1*$^{fl/fl}$ mice. breeding cages will be established to generate mice for this project. Mice will be bread and genotype confirmed using PCR.
1b. Generation of stabilized tibial fractures. We will examine five mice in which the conditional alleles is activated (treated with Ad-cre) and five treated with the control virus at each time point (7, 14, and 28 days post fracture).
1c. Analysis of beta-catenin activity. After the fractures are harvested, the fractures will be examined for beta-catenin activity using Western analysis, immunohistochemistry, and using RNA analysis for the target gene Axin2.

β-catenin is activated during tibial fracture repair in neurofibromatosis

We compared the levels of β-catenin and its transcriptional activation during fracture repair in mice deficient in *Nf1*.

To determine how *Nf1* influences the level of β-catenin, we compared the protein level of β-catenin and the expression of its target gene, *Axin2*, between mutant and wild type mice in the native bone as well as during tibial fracture repair. Since *Nf1*$^{-/-}$ mice are not viable, we used mice in which we can conditionally knockout the *Nf1* gene using Cre-LoxP technology (*Nf1*$^{fl/fl}$ mice)[4]. Stabilized tibial fractures were generated in 3 month old male mice using the techniques we previously reported and used in our work examining β-catenin in fracture healing[2]. The floxed alleles were activated using infection with Ad-cre before and at the time of the fracture. As a control, we used the same virus, but that expresses GFP, rather than cre-recombinase. We, and found that this results in

effective recombination of roughly 90% of cells at the fracture repair site[2]. Five mice from each group were sacrificed at 7, 14, and 28 days post fracture for analysis. The protein level of β-catenin was determined using immunoblot from protein extracted from the fracture site and immunohistochemistry on fracture sections. *Axin2* RNA level was measured by PCR from the fracture site, as previous reported[2, 5]. We found a significant increase in β-catenin and Axin expression at one week following a fracture in mice lacking Nf1 (Fig. 2). Thus showing that in fracture repair, β-catenin is hyperactivated in the initial phases of repair. By four weeks, the level had returned to baseline.

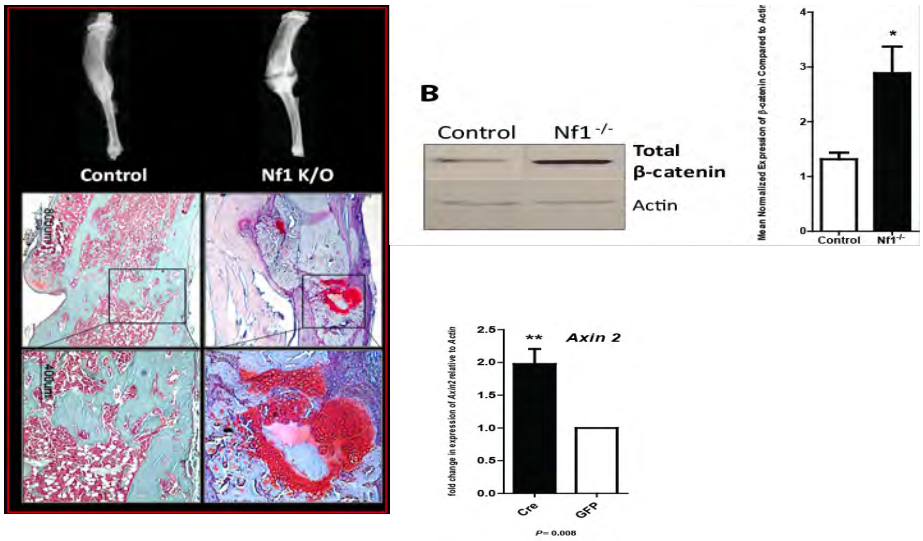

Figure One: Representative western analysis for β-catenin showing an increased protein level during tibial fracture repair in mice lacking Nf1 (Ad-cre) after one week in culture. Densitometry showing the level of β-catenin in the mice at the same time period.

Task 2. Determine how β-catenin modulation alters the healing of fractures in mice deficient in *Nf1* using genetic and pharmacologic manipulation

2a. Generate *Nf1*$^{fl/fl}$; *Catnb*$^{tm2Kem(fl/fl)}$, and *Nf1*$^{fl/fl}$; *Catnb*$^{(+/+)}$ mice *Nf1*$^{fl/fl}$ mice and *Catnb*$^{(+/+)}$ mice will be crossed and then back-crossed to generate *Nf1*$^{fl/fl}$; *Catnb*$^{(+/+)c}$and *Nf1*$^{fl/fl}$; *Catnb*$^{(+/+)}$ mice.
2b. Generation of stabilized tibial fractures. We will examine ten mice *Nf1*$^{fl/fl}$; *Catnb*$^{tm2Kem(fl/fl)}$ mice and ten *Nf1*$^{fl/fl}$; *Catnb*$^{(+/+)}$ mice in which the conditional alleles are activated (treated with Ad-cre) at each time point (7, 14, and 28 days post fracture). We will also examine an additional ten *Nf1*$^{fl/fl}$; *Catnb*$^{(+/+)}$ mice at each time point treated with the control adenovirus.
2c. histological, radiographic, and mechanical analysis The fracture generated in aim 2b will be analyzed radiographically, histologically and mechanically as outlines in the main body of the proposal. This analysis will be undertaken in sequentially as the animals are sacrificed.
2d. Tibia fracture healing will be observed in Nf1fl/fl mice treated with Nefopam or carrier. Mouse fractures identical to those in 1b will be generated, but these mice will be treated with either nefopam or a carrier. We will observe ten mice in each treatment group at each time point. The mice will be treated with Nefopam for the entire duration of the healing process. They will be analyzed as above.

β-catenin inhibition improves the quality of tibial fracture repair in neurofibromatosis

To determine if the NF1 fracture healing phenotype can be rescued in vivo by modulation of β-catenin, mice in which we can conditionally knockout the *Nf1* gene (*Nf1*$^{fl/fl}$ mice) were crossed with mice in which we can stabilize β-catenin. *Catnb*$^{tm2Kem(fl)}$ mice have LoxP sites flanking the first six exons of β-catenin, and when exposed to Cre-recombinase, this results in a knockout of β-catenin. Standard breeding strategies were used to generate *Nf1*$^{fl/fl}$; *Catnb*$^{tm2Kem(fl/fl)}$ and *Nf1*$^{fl/fl}$; *Catnb*$^{(+/+)}$ mice. Importantly, both mice are on a black-6 background and the alleles are on different chromosomes. Three moth old male littermates were compared for how reducing β-catenin protein level alters the fracture repair in the *Nf1* deficient mice. Stabilized tibial fractures weree generated in the mice, as we previously reported. The floxed alleles were activated using infection with Ad-cre as in our previous work. As a control, we used the same virus, but that expresses GFP, rather than cre-recombinase. Ten mice from each group were sacrificed at 7, 14, and 28 days post fracture for radiographic analysis and histology.

Based on our previous work examining β-catenin in fracture repair, we used a power calculation to determine that ten mice in each group at each time frame were selected to allow us to detect a 25% difference in osteoid volume at the fracture site, a clinically significant difference. Safranin-O, Trichrome and H&E staining was performed on the fractures. Quantitative histomorphometry was performed on the sections using the Bioquant Osteo morphometry system.

These mice are still in the process of being analyzed, but our data so far shows a a significant increase in bone formation at the fracture site, when mice lacking Nf1 also were deficient in β-catenin. This was detected as a 25% increase in bone at the fracture site, as detected radiographic and histological analysis at 14 and 28 days after fracture. Micro-CT analysis confirmed a 25% increase in ossification at the fracture repair site in Nf1 deficient mice also deficient in β-catenin ($p<0.01$) compared to Nf1 deficient mice.

Histomorphometry at both time points, showed a 25 and 20% increase in bone volume/ total volume on histologic analysis at the repair site in Nf1 deficient mice also deficient in β-catenin compared to Nf1 deficient mice ($p<0.01$ and $P<0.05$ respectively).

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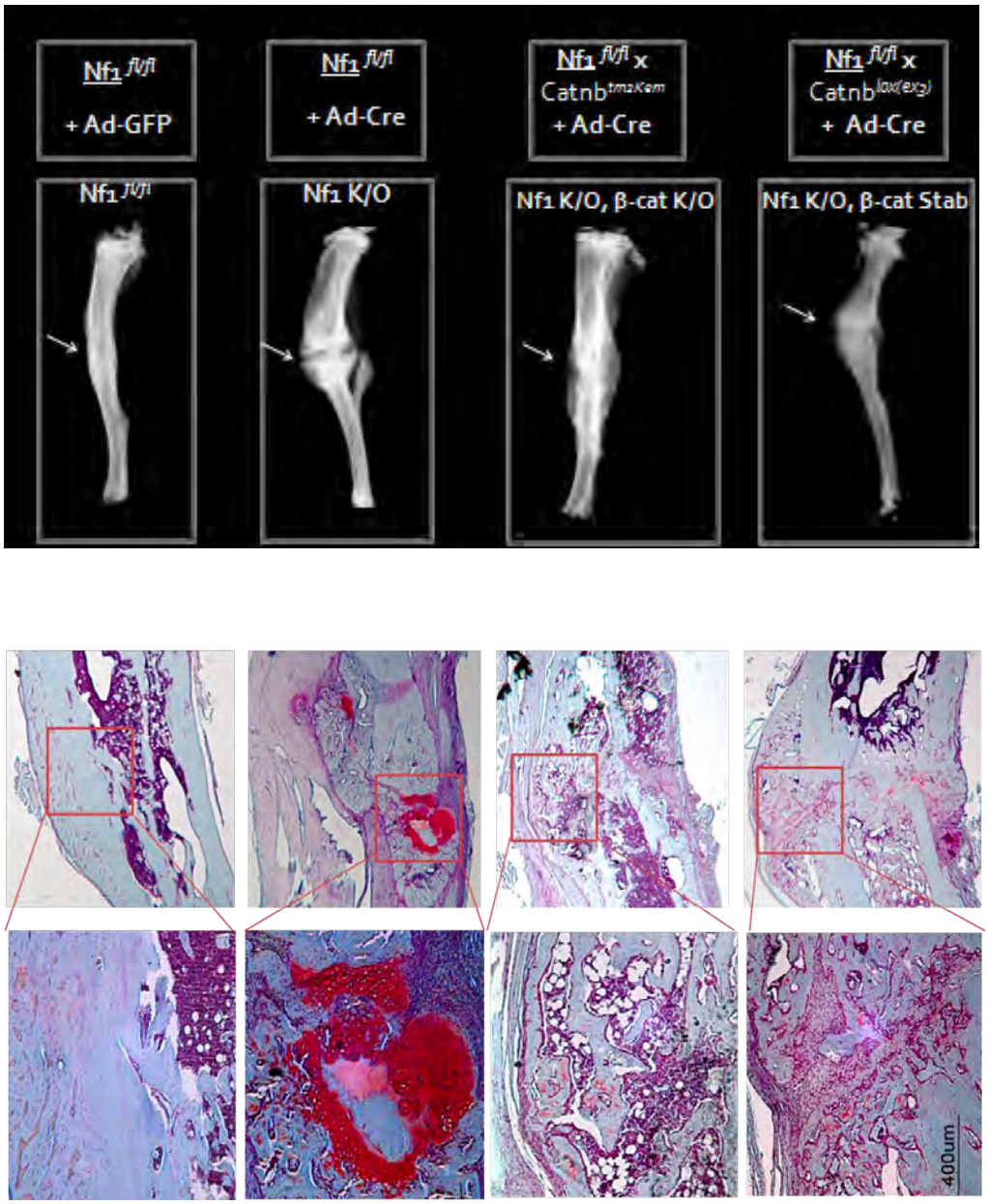

Figure Two: Top. Representative radiographic views of healing tibial fractures in the various geneotypes. There is a delay in formation of bone in Nf1 deficient mice (second panel), which is rescued in mice also deficient in β-catenin (third panel). The arrow shows the fracture site, and an obvious difference in the quality of the union is observed. Bottom shows histologic sections of the same healing fractures, showing a deficiently in bone in the Nf1 deficient facture (Safarin and O stained sections showing increased red

staining for proteogylan – indicating cartilage as opposed to bone). This is rescued in mice also deficient for β-catenin. Histomorphometry confirmed a 25 % increase in bone volume/ total volume on histologic analysis at the repair site in Nf1 deficient mice also deficient in β-catenin compared to Nf1 deficient mice ($p<0.01$).

Decreased osteoblastic differentiation by mutation of the Nf1 gene is ameliorated by Nefopam

Bone marrow of control and Nf1-/- mice was aspirated and adhered to tissue culture plastic. Osteogenic media was used to differentiate bone marrow stromal cells into osteoblasts. Cultures from the bone marrow of Nf1 conditional null mice had significantly lower osteoblastic colonies (ALP stain) and significantly lower mineralized nodules (VK stain). This decrease in osteoblastic potential was remedied rescued when Nf1-/- cells were cultured in the presence of Nefopam. Nf1 cells treated with Nefopam had a higher number of ALP colonies and VK colonies. rt-PCR investigation of differentiating cells showed an increase in osteogenic genes in cultures of control cells but not in cultures of Nf1-/- cells. Treatment with Nefopam led to higher levels of osteogenic transcripts in cultures from Nf1-/- mice. Nefopam treatment of Nf1bone marrow stromal cells led to more robust osteoblastic differentiation and matrix formation and mineralization *in vitro*.

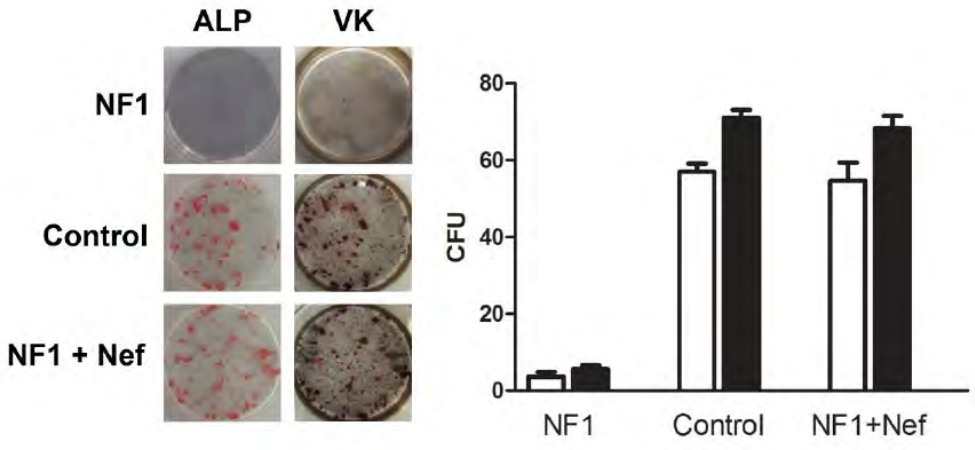

Figure Three: Representative CFU assays showing that treatment of Nf1 deficient mice (labeled NF1) with Nefopam (labeled Nef) brings the numbers of CFU-O (labeled wither with alkaline phosphatase, ALK - while bars; or von kossa, VK - black bars) to levels seen in wild type mice (labeled control).

Impaired fracture repair is rescued by treatment with Nefopam

Control mice and Nf1conditional null mice were subjected to tibial fracture and calluses were harvested at 14- and 21-days post fracture. Safranin-O stained histology of 14-day calluses shows prominent red staining indicative of cartilage deposition and normal healing in the calluses of control mice but not Nf1 mice. This stain is indicative of

cartilage deposition. Indeed histomorphometry of fracture calluses indicates less bone deposition, less cartilage deposition, and greater fibrous tissue deposition in fracture calluses from Nf1 conditional null mice relative to control mice. This deregulation of fracture repair was rescued upon treatment with Nefopam. Nf1 conditional null mice treated with Nefopam had fracture calluses which had more bone, more cartilage, and less fibrotic tissue than untreated mutants. At 21-days post fracture radiographs of fracture calluses from control mice show union while those from Nf1 conditional null mice show non-union however, treatment of Nf1 conditional null mice with Nefopam lead to union by 21-days post fracture. Histomorphometry 21-days post fracture shows less bone and more fibrous tissue in the fracture calluses from Nf1 conditional null mice relative to control. This shortcoming in fracture repair is remedied by treatment of Nf1 conditional null mice with Nefopam.

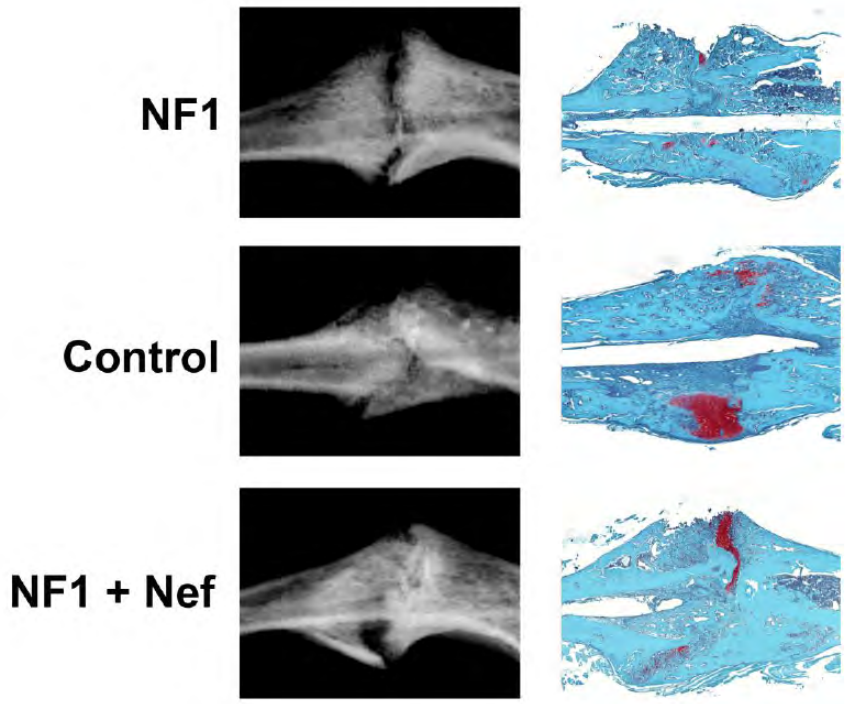

Figure Four: Top. Representative radiographic and histologic views of healing tibial fractures. There is a delay in formation of bone in Nf1 deficient mice (top panels), compared to controls (middle panels). The bottom panels show bone formation in Nf1 deficient mice treated with Nefopam. Histomorphometry confirmed a 20 % increase in bone volume/ total volume on histologic analysis at the repair site in Nf1 deficient mice treated with nefopam to Nf1 deficient mice alone ($p<0.05$).

Key research accomplishments

1) beta-catenin protein level during fracture repair in mice lacking the Nf1 gene measured five times higher than normal.

2) Mice lacking Nf1 gene showed deficient fracture repair, with no osteoblasts at the fracture site 3 weeks after fracture.

3) Mice lacking Nf1 gene, but that also express a low level of beta-catenin healed their tibia fracture quicker and with more bone as measured using both radiographic and histologic parameters.

4) Nefopam treatment improves fracture repair in Nf1 deficient mice.

Reportable outcomes

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Nothing to report

INVENTIONS, PATENTS AND LICENSES:

Nothing to report

REPORTABLE OUTCOMES:

Nothing to report

OTHER ACHIEVEMENTS:

Nothing to report

Conclusions

Beta-catenin protein is elevated during fracture repair in mice lacking Nf1. Inhibition of beta-catenin can improve the quality of the bone repair process.

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